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Mechanisms of the proteinuria induced by Rho GTPases

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Podocytes are highly differentiated cells that play an important role in maintaining glomerular filtration barrier integrity; a function regulated by small GTPase proteins of the Rho family. To investigate the role of Rho A in podocyte biology, we created transgenic mice expressing doxycycline-inducible constitutively active (V14 Rho) or dominant-negative Rho A (N19 Rho) in podocytes. Specific induction of either Rho A construct in podocytes caused albuminuria and foot process effacement along with disruption of the actin cytoskeleton as evidenced by decreased expression of the actin-associated protein synaptopodin. The mechanisms of these adverse effects, however, appeared to be different. Active V14 Rho enhanced actin polymerization, caused a reduction in nephrin mRNA and protein levels, promoted podocyte apoptosis, and decreased endogenous Rho A levels. In contrast, the dominant-negative N19 Rho caused a loss of podocyte stress fibers, did not alter the expression of either nephrin or Rho A, and did not cause podocyte apoptosis. Thus, our findings suggest that Rho A plays an important role in maintaining the integrity of the glomerular filtration barrier under basal conditions, but enhancement of Rho A activity above basal levels promotes podocyte injury.

Kidney International (2012) **81,** 1075–1085; doi:10.1038/ki.2011.472; published online 25 January 2012

KEYWORDS: cell signaling; glomerular disease; podocyte

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Received 4 April 2011; revised 10 October 2011; accepted 8 November 2011; published online 25 January 2012

Podocytes are highly differentiated cells that play an important role in maintaining the integrity of the glomerular filtration barrier.[1–3] Their function is regulated by small GTPases belonging to the Rho GTPase family.[4–6] These small GTPases act as molecular switches controlling activation of multiple downstream effector molecules.[7–10] Among their pleiotropic actions, Rho-dependent signaling cascades modulate cellular morphology and actin polymerization, adhesion, cell migration, proliferation, and apoptosis, as well as participate in contractile responses.[7–10] Although these actions likely serve homeostatic functions under normal physiologic conditions, Rho-dependent signaling cascades are highly activated during inflammatory states, which, in turn, may have pathological consequences.[11–19] In this regard, a large body of data implicate Rho GTPases in the pathogenesis of disease processes in the kidney including glomerular diseases.[11–19] Moreover, a growing literature suggests that Rho A may also play an important homeostatic function by promoting a podocyte phenotype that stabilizes the glomerular architecture.[4–6] In this scenario, some basal level of Rho A activity would be beneficial. In contrast, high levels of Rho A activity induced by inflammatory processes may cause podocyte injury.[11–19] Indeed, recent studies provide strong evidence that enhanced Rho A activity in podocytes has adverse effects on glomerular filtration barrier function.[20] The mechanisms of altered glomerular permselectivity after Rho A activation, however, have not been extensively characterized. Moreover, there is little information on the role of basal Rho A activity in regulating glomerular filtration barrier integrity.

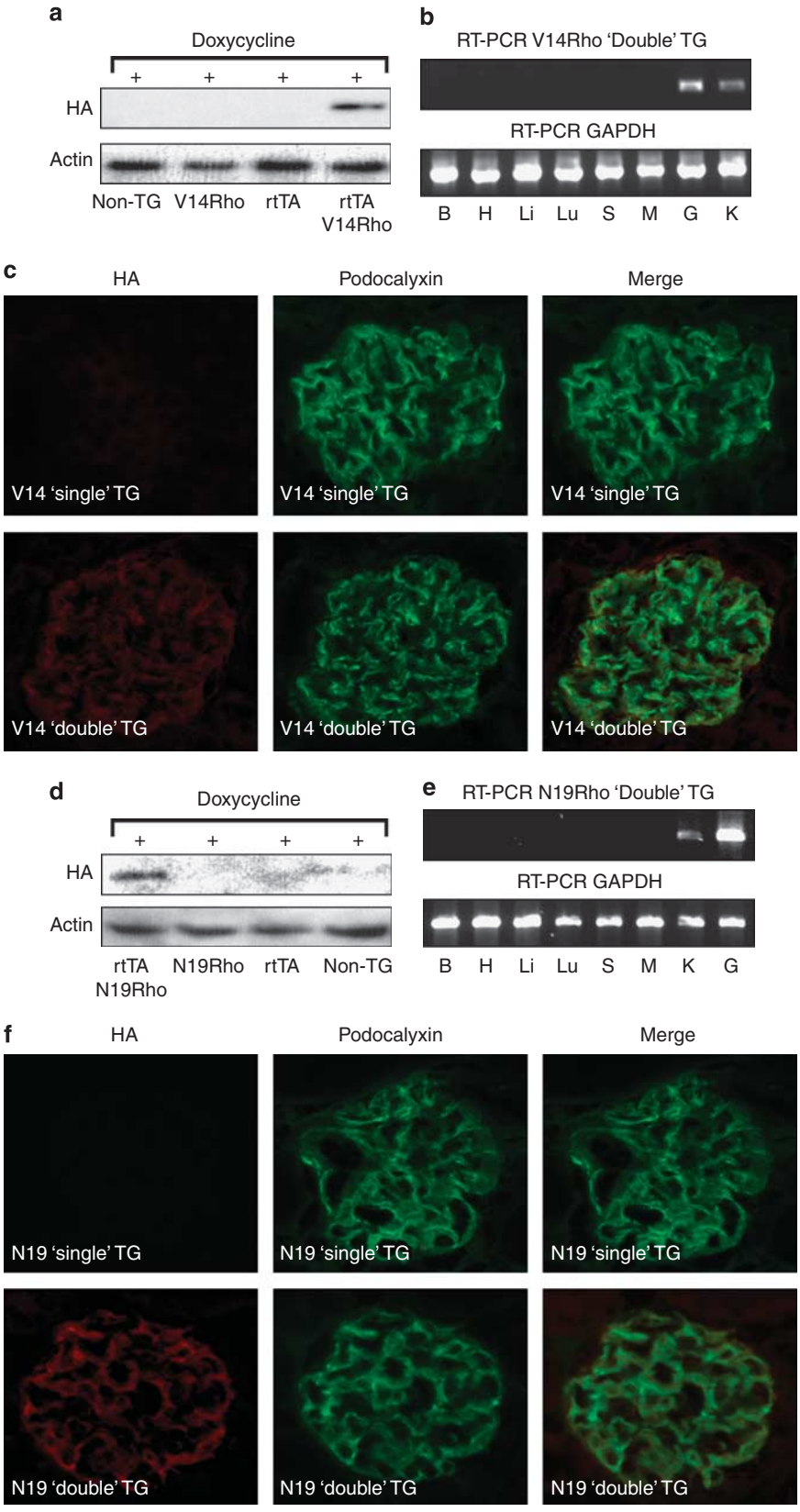
In this study, we investigated the effect of modulating Rho A activity in glomerular podocytes by creating transgenic (TG) mice that expressed either a constitutively active Rho A (V14 Rho) or a dominant-negative Rho A (N19 Rho) specifically in podocytes using a doxycycline-inducible system. Using these TG mice, we found that either activation or inhibition of Rho A in podocytes *in vivo* had adverse effects on podocyte function.

RESULTS

Creation of V14 Rho and N19 Rho TG mice

For the experiments, we utilized the Tet-On system,[21] which requires two TG mice for podocyte-specific expression. The

first TG animal expresses the reverse tetracycline-controlled transcriptional activator (rtTA) under the control of the human podocin (NPHS2) promoter.[22] The second TG mouse expresses either V14 Rho or N19 Rho under the control of tet operator sequence (tetO) and a minimal cytomegalovirus (CMV) promoter (PminCMV).[21] By breeding the two TG



mice, animals are obtained that express both transgenes. In these 'double' TG mice, treatment with doxycycline induces transgene expression.

For the experiments, two independent TG lines were established for each transgene. Figure 1a and d shows expression of the hemagglutinin (HA)-tagged transgenes after 1 week of doxycycline treatment by immunoblotting for the HA epitope using glomerular preparations from 'double' TG mice (rtTA and either the V14 Rho or N19 Rho transgenes) as well as 'single' TG mice (rtTA, v14Rho, or N19 Rho transgenes) and non-TG mice. Expression of either the V14 Rho or N19 Rho protein was detectable by immunoblotting in 'double' TG mice but not in 'single' TG or non-TG mice (Figure 1a and d). In the absence of doxycycline, the Rho proteins were not detected in non-TG or 'single' TG or 'double' TG mice (not shown).

Figure 1b and e shows tissue-specific expression of the V14 Rho and N19 Rho by reverse transcriptase-PCR (RT-PCR) using mRNA prepared from mice treated with doxycycline. As shown in the top panel of Figure 1b and e, a RT-PCR product of the appropriate size was detected in kidney cortex and glomerular preparations from 'double' TG mice. No RT-PCR products were detected in other tissues from the 'double' TG mice. The glyceraldehyde 3-phosphate dehydrogenase RT-PCR reaction confirmed that the reverse transcriptase reaction was successful in the tissues examined (lower panel). In data not shown, expressions of the V14 Rho or N19 Rho transgenes were: (1) not detected by RT-PCR in any of the tissues from other doxycycline-treated 'single' TG mice and non-TG controls, and (2) not detected by RT-PCR in 'double' TG mice in either the absence of doxycycline treatment or in the absence of a RT reaction.

We next determined cell-specific expression of the transgene. For these studies, tissue sections were stained for expression of the HA-tagged V14 Rho or N19 Rho transgenes (rhodamine) and the podocyte marker podocalyxin (fluorescein). As shown in Figure 1c and f, only podocalyxin was detected in 'single' TG mice treated with doxycycline. In contrast, the lower panels show that both the HA epitope and podocalyxin were detected in 'double' TG mice treated with doxycycline. Merging the two images suggested that the HA epitope and podocalyxin shared a similar cellular distribution. In data not shown, the HA epitope was not detected in rtTA 'single' TG mice and non-TG controls in the presence of doxycycline or in 'double' TG mice in the absence of doxycycline.

Effect of transgenes on albuminuria

For these studies, 'double' TG mice and controls ('single' TG and non-TG mice) were treated with doxycycline or vehicle for 2 weeks and before measuring albuminuria. Experimental results were similar using the progeny from the independent lines and these data were combined for the analyses. Induction of either the V14 Rho transgene (Figure 2a) or the N19 Rho transgene (Figure 2b) caused a significant increase in albuminuria in 'double' TG mice compared with controls. As shown in Table 1, similar results were seen when albuminuria data were expressed as μg albumin per mg creatinine. Moreover, the increase in albuminuria after transgene induction was reversible 2–4 weeks after discontinuing doxycycline treatment (Table 2).

We next determined the effects of more prolonged transgene induction on albuminuria. For these studies, mice were treated for 6 weeks with doxycycline, and albuminuria was examined at baseline and 2, 4, and 6 weeks after doxycycline treatment. As shown in Figure 2c, induction of V14 Rho caused a progressive increase in albuminuria over the 6-week treatment period compared with control animals. By the 6-week time point, albuminuria was significantly increased compared with the 2-week time point in V14 Rho 'double' TG mice. Induction of the N19 Rho transgene also caused an increase in albuminuria compared with control animals (Figure 2d). The increase in albuminuria, however, was not significantly different during doxycycline treatment in N19 Rho 'double' TG mice at the three time points examined.

Glomerular histomorphology and glomerular ultrastructure

Light microscopic examination of kidney sections revealed minimal abnormalities (Figure 3a–c). Transmission electron microscopy revealed a few areas of foot process (FP) flattening in the control mice (Figure 3d and g). In contrast, large areas of FP effacement involving the majority of glomeruli available for examination were detected in both V14 Rho (Figure 3e and h) and N19 Rho (Figure 3f and i)

Figure 1 | Creation of transgenic (TG) mice and induction of the transgene. In **a** and **d**, induction of the N19 Rho and V14 Rho transgenes, respectively, was investigated by immunoblotting for the hemagglutinin (HA) epitope in non-TG mice, 'single' TG mice, and 'double' TG mice after doxycycline treatment. Both transgenes were induced by doxycycline in 'double' TG mice but not in control mice. Transgene expression was not detected by immunoblotting in the absence of doxycycline (data not shown). rtTA, reverse tetracycline-controlled transcriptional activator. In **b** and **e**, tissue-specific expression of the transgenes was investigated by reverse transcriptase-PCR (RT-PCR) in 'double' TG mice after treatment with doxycycline using transgene-specific primers. A RT-PCR product was detected in both kidney cortex and isolated glomerular preparations from N19 Rho 'double' TG mice and V14 Rho 'double' TG mice as indicated. No RT-PCR products were detected in other tissues from the 'double' TG mice. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control confirmed that the RT reaction was successful in the tissues examined. No RT-PCR products were obtained in any tissues from 'double' TG mice in either the absence of doxycycline treatment or in the absence of an RT reaction (not shown). Similarly, no RT-PCR products were obtained in any tissues from doxycycline-treated 'single' TG or non-TG mice (not shown). B, brain; G, glomeruli; H, heart; K, kidney cortex; Li, liver; Lu, lung; M, muscle (skeletal); S, spleen. In **c** and **f**, tissue sections were stained for expression of the HA-tagged transgenes and the podocyte marker podocalyxin as indicated. As shown in the upper panels, podocalyxin, but not the HA epitope, was detected in 'single' TG mice treated with doxycycline. In contrast, the lower panel shows that both the HA epitope and podocalyxin were detected in 'double' TG mice treated with doxycycline. When the two images are merged, the HA epitope and podocalyxin had a similar cellular distribution in the 'double' TG mice.

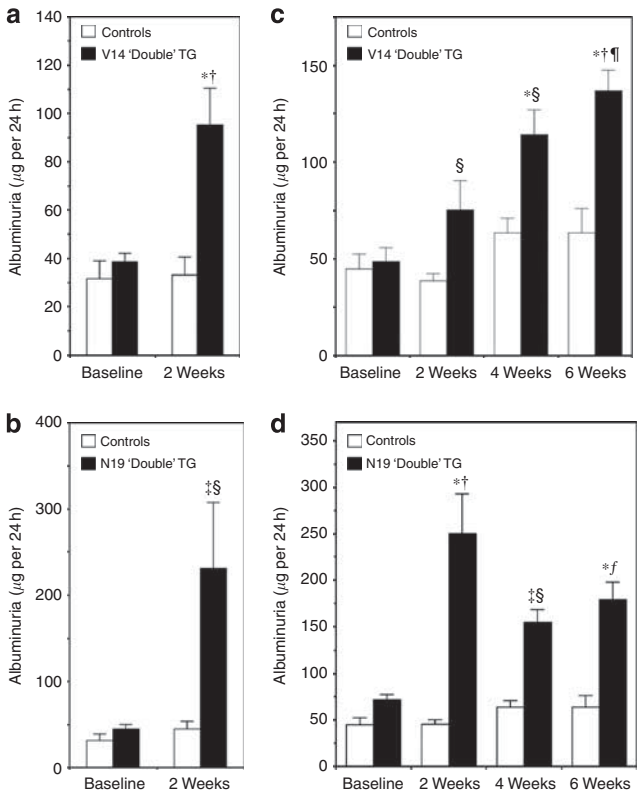


Figure 2 | Effect of transgene induction on albuminuria. In **a** and **b**, albuminuria was measured after 2 weeks of treatment in both N19 Rho 'double' transgenic (TG) mice and V14 Rho 'double' TG mice as well as controls ('single' TG and non-TG mice). Treatment with doxycycline significantly increased albuminuria in (**a**) V14 Rho and (**b**) N19 Rho 'double' TG mice compared with 'double' TG mice at baseline or compared with controls at either baseline or after treatment with doxycycline. In **c** and **d**, mice were treated for 6 weeks with doxycycline and albuminuria was measured at the 2-, 4-, and 6-week time points. (**c**) In V14 Rho 'double' TG mice, albuminuria was increased during doxycycline treatment compared with controls and tended to increase over time. At the 6-week time point, albuminuria was significantly increased compared with the 2-week time point in V14 Rho 'double' TG animals. Albuminuria was also increased in N19 Rho 'double' TG mice compared with controls after doxycycline treatment at all time points examined, but tended to remain relatively stable over the treatment period. Eight to 16 mice were studied per group. $^{\ddagger}P<0.05$ vs. 'double' TG at baseline, $^{*}P<0.01$ vs. 'double' TG at baseline, $^{\S}P<0.05$ vs. controls after treatment with doxycycline, $^{f}P<0.01$ vs. controls after treatment with doxycycline, $^{\dagger}P<0.001$ vs. controls after treatment with doxycycline, $^{\P}P<0.01$ vs. 'double' TG at 2 weeks.

'double' TG mice. To quantitate the severity of FP effacement, we assessed the number of patent slit diaphragms per μm of glomerular basement membrane. There was a significant decrease in the number of patent slit diaphragms per μm of glomerular basement membrane in both V14 Rho 'double' TG mice (2.25 ± 0.21 (controls) vs. 1.24 ± 0.23 (V14 Rho 'double' TG); $P<0.01$) and N19 Rho 'double' TG mice (2.19 ± 0.20 (controls) vs. 1.22 ± 0.25 (N19 Rho 'double' TG); $P<0.01$). The light microscopic and ultrastructural findings were similar in 'double' TG mice and controls after either 2 or 6 weeks of doxycycline treatment.

Table 1 | Albuminuria (μg albumin/mg creatinine)

	V14 Rho studies		N19 Rho studies	
	Controls	'Double' TG	Controls	'Double' TG
Baseline	44 ± 19	25 ± 13	37 ± 11	25 ± 4
Doxycycline	36 ± 8	108 ± 36*,†	36 ± 5	178 ± 54**,†

Abbreviation: TG, transgenic.
$^{*}P<0.025$ or $^{**}P<0.05$ vs. doxycycline-treated controls. $^{\dagger}P<0.05$ vs. 'double' TG at baseline.

Table 2 | Albuminuria (μg albumin per 24 h)

	V14 Rho studies		N19 Rho studies	
	Controls	'Double' TG	Controls	'Double' TG
Baseline	48 ± 6.6	52 ± 7.3	31 ± 6.2	58 ± 9.1
2 Weeks of doxycycline	40 ± 5.2	112 ± 26*,†	27 ± 10	196 ± 61*
2 Weeks after doxycycline	28 ± 19	24 ± 8.6‡	27 ± 10	49 ± 18
4 Weeks after doxycycline	35 ± 19	31 ± 17‡	32 ± 19	11 ± 7.5‡

Abbreviation: TG, transgenic.
$^{*}P<0.001$ vs. doxycycline-treated controls.
$^{\dagger}P<0.01$ vs. baseline V14 'double' TG mice.
$^{\ddagger}P<0.05$ vs. 'double' TG after 2 weeks of doxycycline treatment.

Effect of the transgenes on actin polymerization

Rho GTPases are important regulators of actin polymerization,[7] and maintenance of the actin cytoskeleton plays a critical role in sustaining glomerular filtration barrier function.[4–6] To investigate the effect of Rho A on actin polymerization, we introduced V14 Rho or N19 Rho into cultured podocytes using protein transduction[23] by tagging the proteins with the TAT (transactivator of transcription) human immunodeficiency virus protein sequence (V14 Rho(+) or N19 Rho(+)). Cell-impermeable proteins lacking the TAT sequence were used as controls (V14 Rho(−) or N19 Rho(−)). For the experiments, cultured podocytes were treated with V14 Rho or N19 Rho TAT proteins and then actin polymerization was monitored by phalloidin staining (rhodamine). As shown in Figure 4a, a few stress fibers were seen in podocytes treated with a control Rho A protein lacking the TAT sequence. In podocytes treated with V14 Rho(+), the number of stress fibers was increased (Figure 4b), whereas in cells treated with N19 Rho(+), the number of stress fibers was reduced (Figure 4c). Quantitation of actin polymerization (F actin) is shown in Figure 4d. V14 Rho(+) induced a significant increase in actin polymerization compared with control podocytes. In contrast, actin polymerization was significantly decreased by treatment with N19 Rho(+) compared with either control podocytes or podocytes treated with V14 Rho(+). Podocyte morphology was similar in untreated podocytes and podocytes treated with control TAT proteins (not shown).

Effect of the transgenes on synaptopodin expression

Synaptopodin is an actin-associated protein that is expressed exclusively by podocytes in the kidney.[24] To determine if the effects of the transgenes on actin polymerization (Figure 4) affected synaptopodin expression *in vivo*, we first measured glomerular synaptopodin levels by immunoblotting in mice treated with doxycycline for 6 weeks. As shown in Figure 5a, synaptopodin levels were decreased by induction of either

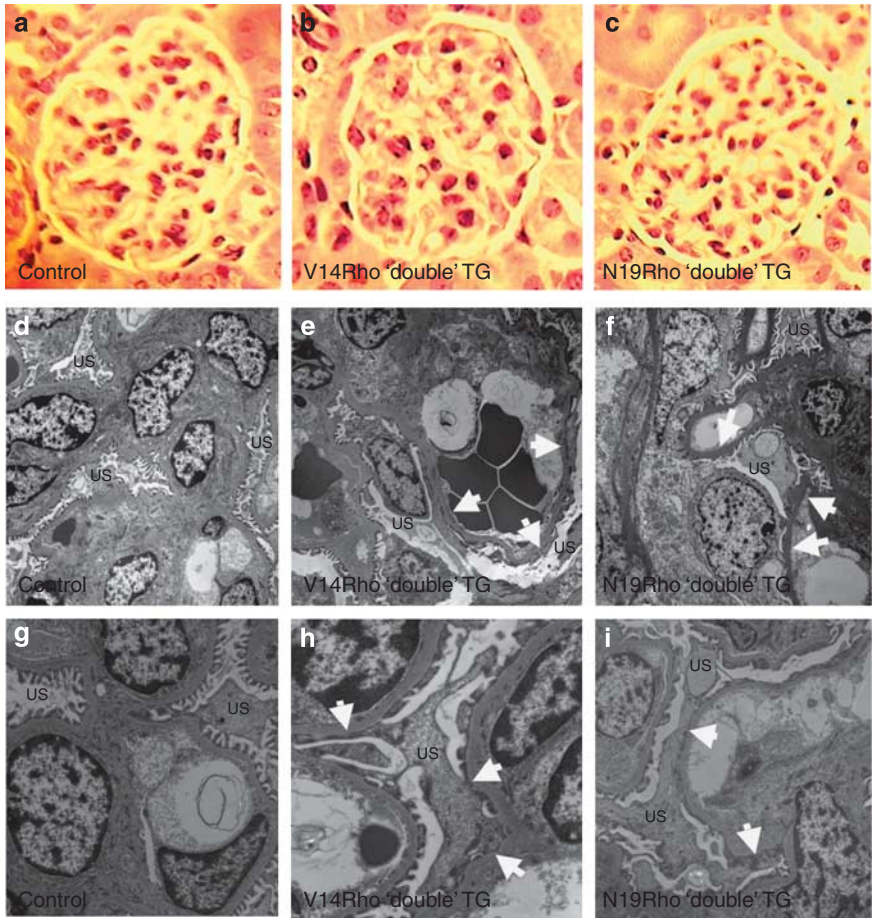


Figure 3 | Renal histology. Glomerular histology in (**a**) controls, (**b**) V14 Rho 'double' transgenic (TG) mice, and (**c**) N19 Rho 'double' TG mice 2 weeks after treatment with doxycycline. Glomerular histology was similar in all groups at the light microscopic level. (**d–i**) Glomerular ultrastructure in controls, V14 Rho 'double' TG mice, and N19 Rho 'double' TG mice 2 weeks after treatment with doxycycline. Low-power views are shown in **d–f** and higher-magnification images are shown in **g–i**. In both N19 Rho and V14 Rho 'double' TG mice, large areas of foot process (FP) effacement (arrows) were detected involving ~40% of the glomerular basement membrane (GBM) length. In contrast, FPs were well preserved in control mice treated with doxycycline, although a few areas of FP flattening were observed. US, urinary space.

V14 Rho or N19 Rho compared with controls. The podocyte protein podocalyxin was not affected by induction of either transgene. Quantitation of synaptopodin protein and mRNA levels is shown in Figure 5b and c, respectively. Synaptopodin protein levels were decreased at both 'double' TG lines compared with controls. A similar pattern was seen for synaptopodin mRNA levels at the 6-week time point. Despite the marked reduction in synaptopodin protein levels by immunoblotting, synaptopodin was detectable by immunohistochemistry in both control mice and 'double' TG mice (Figure 5d), although the intensity of the immunofluorescence tended to be decreased in 'double' TG animals. In V14 Rho 'double' TG mice, synaptopodin staining also tended to be more granular (Figure 5d, bottom panel) compared with the linear staining observed in N19 Rho 'double' TG mice and controls (Figure 5d, top and middle panels). We also investigated synaptopodin expression at the 2-week time point. As shown in Supplementary Figure S1 online, synaptopodin mRNA and protein levels were both decreased in N19 Rho 'double TG mice after 2 weeks of doxycycline treatment. In contrast, synaptopodin mRNA and protein levels were better preserved at this time point in V14 Rho 'double' TG mice, suggesting that the tempo of synaptopodin loss was delayed in these animals.

Effect of the transgenes on expression of Rho A

The Rho GTPase family includes members belonging to the Rho, Rac, and cdc42 GTPases.[25] In podocytes, the Rho A protein is stabilized by interacting with the podocyte protein synaptopodin.[5] We, therefore, determined if decreased synaptopodin levels in 'double' TG mice affected expression of Rho GTPase family members in enriched glomerular preparations after 6 weeks of doxycycline treatment. As shown in Figure 6a and b, Rho A levels were unaffected by transgene induction in N19 Rho 'double' TG animals. In contrast, Rho A levels were significantly decreased in V14 'double' TG mice compared with either controls or N19 Rho 'double' TG animals. As shown in the inset to Figure 6b, the changes in Rho A protein levels represented endogenous Rho A expression because longer exposure of the immunoblots detected expression of the slightly larger Rho A transgenes. Rac1 levels were not changed by transgene induction (Figure 6a) and cdc42 was difficult to detect in the glomerular preparations (not shown).

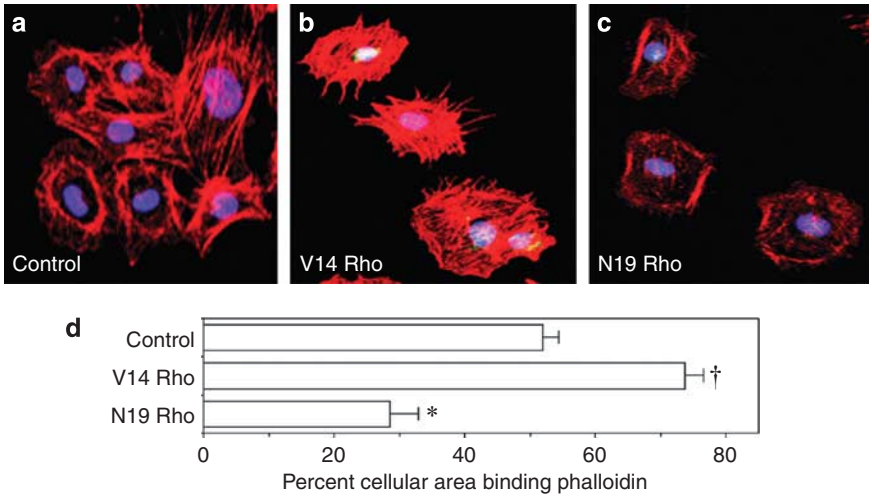


Figure 4 | Effect of V14 Rho and N19 Rho on actin polymerization in podocytes. (**a**) A few stress fibers are seen in podocytes treated with control proteins. (**b**) In contrast, V14 Rho(+) treatment enhanced the number of stress fibers, whereas (**c**) treatment with N19 Rho(+) caused a decrease in stress fiber formation. Quantitation of the percent cellular area binding phalloidin is shown in **d**. There was a significant increase in the percent cellular area binding phalloidin in V14 Rho(+)-treated podocytes compared with cells treated with either control proteins or N19 Rho(+). Treatment with N19 Rho(+) significantly decreased phalloidin binding compared with cells treated with either control proteins or V14 Rho(+). *$P<0.01$ vs. cultured podocytes treated with either control TAT (transactivator of transcription) proteins or N19 Rho(+), †$P<0.01$ vs. cultured podocytes treated with either control TAT proteins or V14 Rho(+).

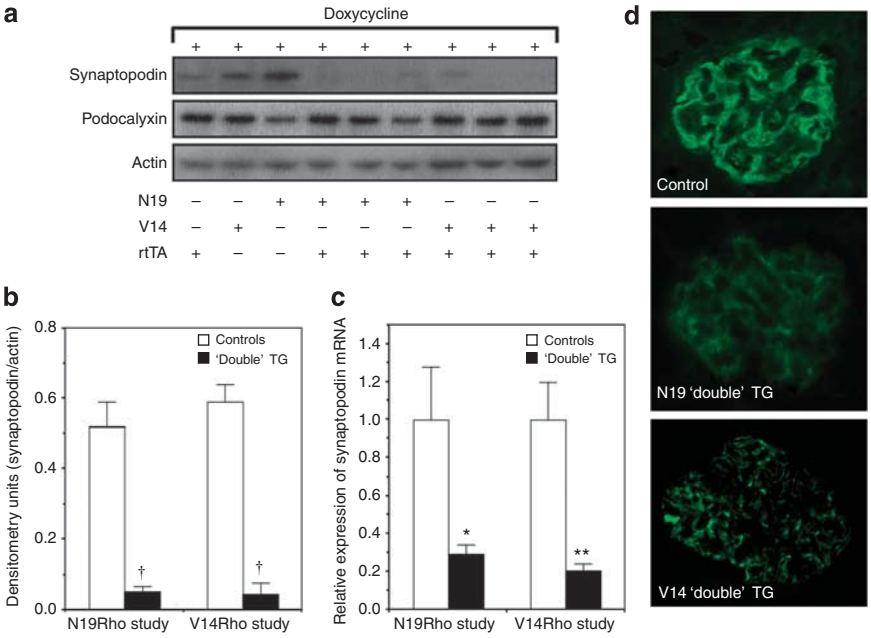


Figure 5 | Effect of the Rho transgenes on synaptopodin and podocalyxin expression. (**a**) Representative immunoblots of synaptopodin and podocalyxin protein levels in enriched glomerular preparations after 6 weeks of doxycycline treatment. Synaptopodin levels were difficult to detect in both N19 Rho and V14 Rho 'double' transgenic (TG) mice compared with controls. In contrast, podocalyxin levels were not changed in any of the groups. rtTA, reverse tetracycline-controlled transcriptional activator. Quantitation of the immunoblots is shown in **b**. Induction of the Rho A transgenes in 'double' TG mice significantly reduced glomerular synaptopodin protein levels compared with controls. As shown in **c**, a similar reduction in synaptopodin mRNA levels was seen in both N19 Rho and V14 Rho 'double' TG mice compared with controls. In **d**, synaptopodin was detected by immunohistochemistry in all groups, although the immunofluorescence tended to be reduced in the 'double' TG mice. Moreover, the staining appeared more granular in the V14 'double' TG animals. Four to 6 samples were studied per group. *$P<0.05$ or **$P<0.01$ vs. controls, †$P<0.001$ vs. controls.

Effect of Rho A on podocyte apoptosis

Rho A plays an important role in regulating cell death, in part, by inhibiting the prosurvival and anti-apoptotic phophatidylinositol-3-kinase (PI3K)/Akt pathway.[25–27] This negative regulatory effect is mediated by the downstream Rho A effectors Rho kinases or ROKs,[25] which phosphorylate and activate PTEN (phosphatase and tensin homolog deleted on chromosome ten).[28] PTEN, in turn, dephosphorylates and

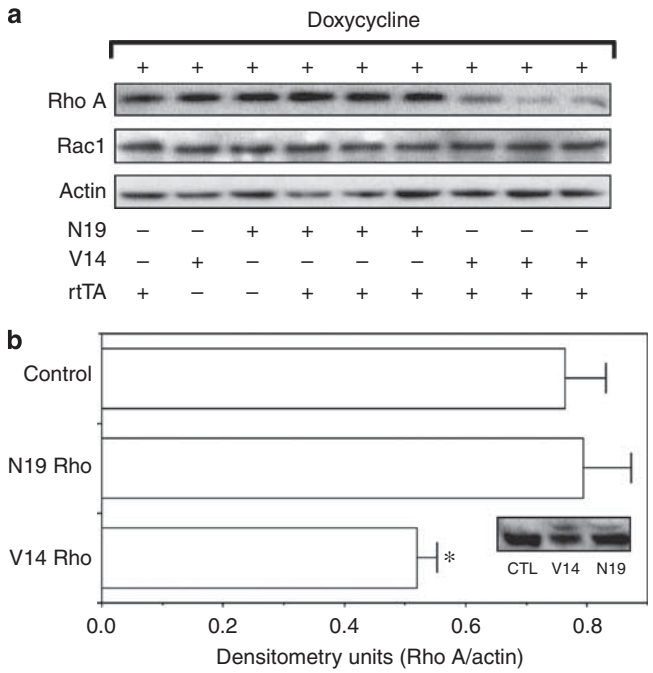


Figure 6 | Effect of the Rho transgenes on expression of Rho GTPase family members. In **a**, Rho A levels were decreased in V14 Rho 'double' transgenic (TG) mice compared with controls. In contrast, Rac1 expression was similar in all groups examined. rtTA, reverse tetracycline-controlled transcriptional activator. Quantitation of the Rho A immunoblots is shown in **b**. Induction of the V14 Rho transgene in 'double' TG mice significantly reduced glomerular Rho A protein levels compared with controls as well as N19 Rho 'double' TG mice. The changes in Rho A protein levels represented endogenous Rho A expression because longer exposure of the immunoblots detected expression of the slightly larger Rho A transgenes (inset). Nine samples were studied per group, $^*P < 0.05$ vs. V14 Rho controls or N19 Rho 'double' TG mice.

inactivates Akt.[25] To investigate the effect of Rho A on podocyte apoptosis *in vitro*, we treated cultured podocytes with V14 Rho or N19 Rho TAT proteins and then measured apoptosis as described in the Materials and Methods section. As shown in Figure 7a, V14 Rho(+) enhanced podocyte apoptosis compared with V14 Rho(−), and this apoptotic effect was blocked by the Y27632. In contrast, N19 Rho(+) had no significant effect on apoptosis of cultured podocytes compared with cells treated with N19 Rho(−) (7.4 ± 3.2 (N19 Rho(+)) vs. 7.2 ± 1.7 (N19 Rho(−)) percent apoptosis above baseline; P = NS). To assess PTEN phosphorylation levels, we immunoprecipitated PTEN and then immunoblotted for PTEN and phosphothreonine (Figure 7b). Densitometric quantitation of the immunoblots is shown in Figure 7c. Phospho-PTEN levels were significantly increased in cultured podocytes 10 and 30 min after treatment with V14 Rho(+) compared with cells treated with V14 Rho(−).

To determine if Rho A activation caused podocyte apoptosis *in vivo*, we assessed both podocyte number and podocyte apoptosis after 6 weeks of doxycycline treatment. We were, however, unable to detect podocyte apoptosis, and the number of podocytes per glomerular profile was similar in controls and either N19 Rho 'double' TG mice (8.1 ± 0.4 (controls) vs. 8.5 ± 0.5 (N119Rho); P = NS) or V14 Rho 'double' TG mice (8.4 ± 0.2 (controls) vs. 8.1 ± 0.2 (N119Rho); P = NS).

Effect of transgene induction on glomerular nephrin expression

The podocyte protein nephrin plays a critical role in maintaining the integrity of the glomerular filtration barrier,[29–31] and its expression is decreased in some,[32–40] although not all,[32] proteinuric glomerular diseases. In addition to its structural role, nephrin is also a signaling molecule.[27,41,42] In this regard, nephrin may inhibit podocyte apoptosis by activating the prosurvival PI3K/Akt pathway.[25,41] In this scenario, a decrease in glomerular nephrin expression might promote apoptosis of glomerular podocytes. We, therefore, measured nephrin mRNA levels using quantitative RT-PCR and glomerular mRNA prepared from mice treated for 6 weeks with doxycycline. As shown in Figure 8a, induction of the V14 Rho transgene caused a reduction in nephrin mRNA levels. In contrast, nephrin mRNA levels were similar in N19 'double' TG mice compared with controls. Figure 8a and b shows representative immunoblots of nephrin protein levels in glomerular preparations from N19 Rho and V14 Rho 'double' TG mice and controls ('single' TG and non-TG mice) treated for 6 weeks with doxycycline. Quantitation of the immunoblots is shown in Figure 8d. There was a significant decrease in nephrin protein levels in V14 Rho 'double' TG compared with controls. In contrast, nephrin protein levels were similar in N19 Rho 'double' TG compared with control animals. Similar results were obtained at the 2-week time point (Supplementary Figure S2 online). With longer exposure nephrin protein appears as a doublet.

DISCUSSION

In this study, we found that both Rho A activation and Rho A inhibition, specifically in glomerular podocytes, caused albuminuria and FP effacement. These data, taken together with recently published studies,[20] suggest that Rho activity in podocytes must be tightly regulated to maintain podocyte function. Given that excessive Rho GTPase activity likely contributes to glomerular damage in disease states,[11–19] the observation that Rho A inhibition also promotes podocyte injury has implications for treatment strategies in glomerular disease processes. In this regard, chemically distinct ROK inhibitors attenuate renal damage in a variety of experimental models.[11–19] Moreover, ROK inhibition reduces glomerular injury in animal models without adversely affecting glomerular filtration barrier function.[11–15,18,19] In contrast, Rho A inhibition caused albuminuria and FP effacement in this study. Although we cannot exclude 'off-target' effects of transgene overexpression, these data are consistent with the notion that treatment strategies that inhibit Rho A may be difficult to employ clinically because of the potential for damaging the integrity of the glomerular filtration barrier, whereas strategies that target downstream effectors of Rho A such as ROKs may attenuate the detrimental effects of

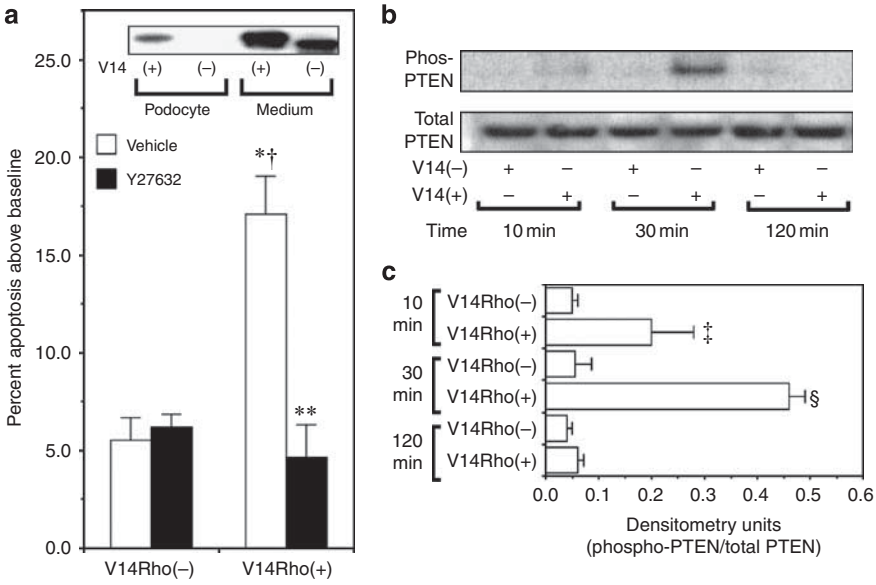


Figure 7 | Effect of V14 Rho(+) on podocyte apoptosis. In **a**, treatment with V14 Rho(+) significantly increased podocyte apoptosis compared with cells treated with V14 Rho(−), and Y27632 significantly reduced podocyte apoptosis induced by V14 Rho(+). Immunoblotting of the hemagglutinin (HA)-tagged V14 Rho proteins in either culture medium or podocyte lysates is shown in the inset. The HA-tagged V14 Rho(+) was effectively transduced into cultured podocytes. There is a slight difference in molecular size of V14 Rho(+) and V14 Rho(−) due to the presence or absence of the TAT (transactivator of transcription) sequence. In **b**, total PTEN (phosphatase and tensin homolog deleted on chromosome ten) and phospho-PTEN levels were assessed in immortalized podocytes after treatment with V14 Rho(+) or V14 Rho(−). Densitometric quantitation of the immunoblots is shown in **c**. Phospho-PTEN levels were significantly increased in cells treated with V14 Rho(+) at the 10- and 30-min time points. Four to 7 samples were studied per group for the apoptosis experiments. Four to 5 samples were studied per group for the immunoblotting studies. *$P<0.001$ vs. cells treated with V14 Rho(−) and vehicle, $^{\dagger}P<0.01$ vs. cells treated with V14 Rho(−) and Y27632, **$P<0.001$ vs. cells treated with V14 Rho(+) and vehicle $^{\ddagger}P<0.05$ or $^{\S}P<0.01$ vs. cells treated with V14 Rho(−).

excessive Rho A activity in glomerular diseases without adversely affecting glomerular filtration barrier function.

Although both Rho A activation and Rho A inhibition promoted albuminuria and FP effacement, the mechanisms appeared to be different. For example, V14 Rho reduced glomerular nephrin expression, increased the number of stress fibers, and promoted podocyte apoptosis. In contrast, N19 Rho had little effect on glomerular nephrin levels or podocyte apoptosis but reduced stress fiber formation. These findings suggest that the mechanisms of proteinuria induced by Rho A activation are different from the mechanisms that promote proteinuria during Rho A inhibition. In this regard, a major function of glomerular podocytes is to provide structural support to the glomerular tuft.[43] By modulating actin polymerization, Rho GTPases likely play a pivotal role in regulating podocyte morphology that, as growing literature suggest, is important for maintaining the integrity of the glomerular filtration barrier.[4–6] In proteinuric renal diseases, a reduction in the activity of Rho GTPases may lead to a phenotypic change in the glomerular podocyte characterized by a loss of stress fibers and enhanced formation of lamellipodia and filopodia as a result of activation of the Rho GTPase family members Rac1 and cdc42.[4–6] Indeed, in humans with minimal change disease, there are few light microscopic abnormalities, but the podocyte undergoes a phenotypic switch characterized by FP effacement and, in some patients, microvillus protrusions on the apical surface reminiscent of filopodia.[32] Despite this apparent change in podocyte morphology, expression of podocyte proteins such as nephrin[32] are not changed in minimal change disease, with the exception of the actin-associated protein synaptopodin that may be decreased.[44] Although we did not detect microvillus protrusions in this study, the N19 Rho 'double' TG mice did exhibit: (1) normal light microscopic findings, (2) FP effacement, and (3) preservation of nephrin expression despite a significant decrease in glomerular synaptopodin levels. Thus, activation of the N19 Rho transgene resulted in many of the features of patients with minimal change disease.

In contrast to Rho A inhibition, enhanced Rho A activity stimulated actin polymerization as well as reduced glomerular nephrin expression and caused apoptosis of cultured podocytes. Although we were unable to demonstrate that induction of V14 Rho in podocytes caused either apoptosis or a decrease in podocyte number *in vivo*, in glomerular diseases, podocyte apoptosis is an important mechanism leading to a decrease in podocyte number.[32,43,45–47] Although the mechanisms that promote podocyte apoptosis are likely complex, the prosurvival PI3K/Akt pathway protects podocytes from apoptosis;[25–27] and PI3K activity is negatively regulated by Rho A through its downstream effector ROK by phosphorylating and activating the Akt inhibitor PTEN.[25] Moreover, the decrease in glomerular nephrin expression following induction of the V14 Rho transgene may also promote apoptosis of glomerular podocytes because nephrin directly stimulates the prosurvival PI3K/Akt pathway.[25,41]

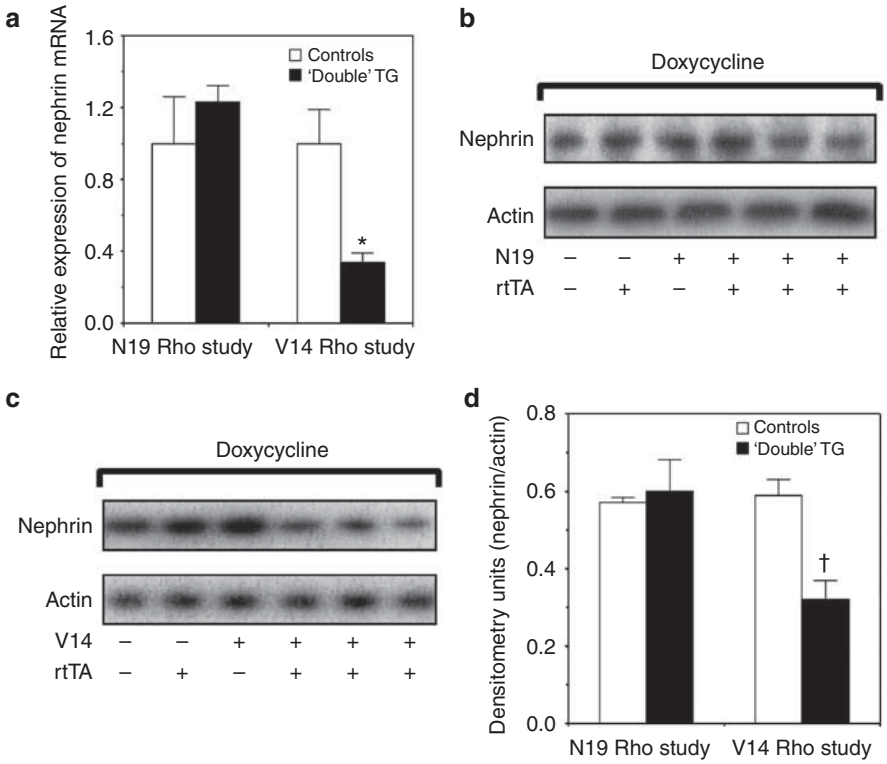


Figure 8 | Effect of the V14 Rho and N19 Rho transgene glomerular nephrin expression. In **a**, induction of the V14 Rho transgene in 'double' transgenic (TG) mice significantly reduced glomerular nephrin mRNA levels compared with controls. (**b**, **c**) Representative immunoblots of nephrin protein levels in enriched glomerular preparations from N19 Rho and V14 Rho 'double' TG mice and controls. rtTA, reverse tetracycline-controlled transcriptional activator. Quantitation of the immunoblots by densitometry is shown in **d**. Induction of the V14 Rho transgene in 'double' TG mice significantly reduced glomerular nephrin protein levels compared with controls. Four to 6 samples were studied per group. $^{*}P<0.01$ vs. V14 Rho controls, $^{\dagger}P<0.05$ vs. V14 Rho controls.

Given that cellular outcome is likely dependent on differential activation of signaling pathways that promote either cell survival or apoptosis, a decrease in PI3K/Akt signaling might alter this balance and promote podocyte apoptosis that, some investigators contend, may cause instability of the glomerular tuft and glomerulosclerosis.[32] Indeed, recent studies suggest that high levels of Rho activation in podocytes promote focal glomerulosclerosis,[20] whereas lower levels of Rho activation cause FP effacement without light microscopic abnormalities.[20] Although we did not observe glomerulosclerosis in our study, it is possible that the level of Rho activation in our experiments may have been insufficient to promote sclerosis. Alternately, differences in experimental design including genetic background of the mice may have contributed to the lack of glomerulosclerosis observed in the present experiments compared with published studies.[20]

Last, both Rho A activation and Rho A inhibition caused a reduction in glomerular synaptopodin expression in 'double' TG mice after 6 weeks of treatment with doxycycline. Synaptopodin stabilizes Rho A protein levels by a protein interaction between Rho A and synaptopodin that, in turn, prevents Rho A from interacting with the ubiquitin ligase Smurf1 and proteosomal degradation.[5] We, therefore, investigated the effect of decreased synaptopodin expression on the level of Rho GTPase proteins. We found that endogenous Rho A protein levels were decreased in V14 Rho 'double' TG mice, consistent with the notion that synaptopodin stabilizes the Rho A protein. In contrast, Rho A protein levels were not affected by expression of the N19 Rho transgene despite a decrease in synaptopodin levels in N19 Rho 'double' TG mice. Although we can only speculate on mechanism, the protein–protein interaction between Rho A and Smurf1 preferentially favors an interaction between inactive Rho A proteins (guanosine diphosphate-bound Rho A) such as the dominant-negative N19 Rho construct.[48] Indeed, V14 Rho binds Smurf1 poorly or not at all.[48] Based on these observations, we speculate that induction of N19 Rho competes with endogenous inactive Rho A proteins (guanosine diphosphate-bound Rho A) and, in turn, competitively antagonizes degradation of endogenous Rho A in N19 Rho 'double' TG mice. This competitive antagonism does not occur in the V14 Rho 'double' TG mice and, as a result, a decrease in synaptopodin levels in these animals enhances degradation of endogenous Rho A.

In summary, we found that either Rho A activation or Rho A inhibition had similar adverse effects on glomerular filtration barrier function and reduced podocyte synaptopodin expression, but the mechanisms of these detrimental effects appeared to be different. Enhanced Rho A activity increased actin polymerization as well as caused a reduction in glomerular nephrin expression and promoted podocyte apoptosis. In contrast, inhibition of Rho A caused a loss of podocyte stress fibers but did not alter glomerular nephrin

expression and did not cause podocyte apoptosis. These data suggest that some basal level of Rho A activity has beneficial effects on podocytes, perhaps by stabilizing the glomerular architecture. The level of Rho A activity, however, must be tightly regulated because enhancing Rho A activity above basal levels also has adverse effects on glomerular filtration barrier function. Taken together with published studies,[11–20] these findings suggest that Rho A plays an important role in modulating the integrity of the glomerular filtration barrier, both under basal conditions and during disease states.

MATERIALS AND METHODS

Materials

Alexa Fluor 568 phalloidin was obtained Molecular Probes (Eugene, OR), the urine albumin and creatinine kits were obtained from AssayPro (St Charles, MO) and Exocell (Philadelphia, PA), respectively, the ROCK inhibitor Y27632[38] was obtained from Calbiochem (La Jolla, CA), and the TAT protein DNA constructs[49] were obtained from Becker-Hapak (Washington University, St Louis, MO).[23]

Creation of inducible V14 Rho and N19 Rho transgenes

The V14 Rho and N19 Rho transgenes were created by subcloning a fragment of the HA-tagged TAT constructs into a previously described construct.[50]

PCR

Screening for TG mice and expression of transgene mRNA were performed using PCR or RT-PCR, respectively, and the primer pairs 5′-AAGGACCAGTTCCCAGAGGT-3′ and 5′-GAAATTGGACAGCA AGAAAG-3′.

Experimental procedures

The following experimental procedures were performed as previously described:[37,38,50–53] (1) culture of mouse podocytes and treatment with TAT proteins, (2) creation of FVB/NJ TG mice and induction of the transgenes, (3) light, electron microscopy and quantitation of slit diaphragm patency, (4) isolation of glomerular preparations, (5) immunoblotting and immunohistochemistry, (6) detection of apoptosis (*in vivo* and *in vitro*), (7) quantitation of glomerular podocytes, (8) immunoprecipitation of PTEN, (9) real-time quantitative RT-PCR, and (10) quantitation of polymerized actin in cultured podocytes. All animal procedures were approved by the Animal Care and Use Committee of Duke University Medical Center. Additional details are provided in the Supplementary Section.

Statistical analysis

Data are presented as the mean ± s.e.m. For comparison between two groups, statistical significance was assessed by a *t*-test using the InStat computer program (GraphPad Software, San Diego, CA). For comparisons between more than two groups, statistical analysis was performed using a one-way analysis of variance followed by a Bonferonni multiple comparisons post test[54] using the InStat program.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

These studies were supported by a Merit Review from Veterans Health Administration, Office of Research and Development (BX000791-01). RFS also received salary support through a grant from the National Institutes of Health, National Institute of Diabetes, Digestive and Kidney Diseases (RO1-DK075688 and RO1-DK087707).

SUPPLEMENTARY MATERIAL

Figure S1. Effect of transgene induction on synaptopodin expression.

Figure S2. Effect of the V14 Rho and N19 Rho transgene glomerular nephrin expression.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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